SUMMARY

1. A method based on the quantitative formation of volatile aldehydes from α -alanine, valine, leucine, isoleucine and possibly other isomers, phenylalanine and methionine ($R \cdot CH(NH_2) \cdot COOH \rightarrow R \cdot CHO$) has been used for the quantitative micro-determination of these amino-acids as a group ('volatile aldehyde amino-acids'). With other amino-acids found in proteins no formation of volatile

aldehydes was detected under our experimental conditions.

2. Acetaldehyde can be determined separately from the mixture of aldehydes. Thus it is possible to determine by means of volatile aldehydes (a) the above group of amino-acids, and (b) alanine. If phenylalanine and methionine are determined separately by specific methods, valine, leucine and isoleucine remain to be determined. Separate determination of the aldehydes formed from them has not yet been successful.

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Experiments on the Localization and Nature of Tea Oxidase

By LIEN PIAO LI (Fellow of the International Training Administration),
Tea Station (Meitan, Kweichow) of the National Agricultural Research Bureau,
Ministry of Agriculture and Forestry, Republic of China,

AND J. BONNER

William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology,
Pasadena, California

(Received 8 August 1946)

The conversion of green tea leaf into the product known as black tea involves the oxidation by leaf enzymes of the phenolic tannins of the leaf, the oxidation being initiated by the crushing of the wilted leaf. Ever since the recognition of the tea fermentation (as the process is generally known) as an enzymic oxidation, attempts have been made to identify the enzyme involved with one or another of the known oxidases. Early workers, notably Mann (1901), Bernard & Welter (1911) and Manskaya (1935) have suggested identity of the tea fermentation enzyme with peroxidase, while tyrosinase (Potapov, 1934) and ascorbic acid oxidase have also been considered (Roberts, 1939a, b). Recent investigations carried on by Lamb & Sreerangachar (1940) in Ceylon and by Roberts and Roberts & Sarma (1938-42) in India have shown, however, that the tea enzyme is neither peroxidase nor ascorbic acid oxidase. Thus the simultaneous presence in the

tea leaf of both peroxidase and the tea tannin does not suffice for fermentation. Ascorbic acid oxidase, if it is indeed present in the leaf at all, does not participate in the fermentation either directly or indirectly. According to Roberts (1942) tea leaf contains a cytochrome oxidase which is capable of oxidizing (presumably through the intermediary of cytochrome) tea tannin, catechol and *p*-phenylenediamine. The workers in India further believe that this same oxidase is the terminal oxidase of the normal respiration of the tea leaf. The views of Roberts and his group (1938-42) are mainly based on experiments carried out with whole minced leaves and these crude enzyme preparations always contained the tea tannin so that substrate specificity of the enzyme could not be clearly studied. The Ceylon workers, on the other hand, have shown (Lamb & Sreerangachar, 1940; Sreerangachar, 1941, 1943a, b, c, d) that the tea enzyme is a polyphenol

oxidase which is highly specific and which oxidizes *o*-polyphenols. Their work was done with purified enzyme preparations free of endogenous substrate. Recent work of Sreerangachar has also shown that the tea oxidase as it exists in the leaf is insoluble and appears to be associated with the solid portion of the cell. Some soluble oxidase activity could, however, be obtained by repeated extraction with buffer of acetone-extracted dried ground leaves. The soluble enzyme contains copper and the activity of purified preparations is proportional to their copper content (Sreerangachar, 1943*d*).

The present work is concerned with a further study of the nature of the tea oxidase, the location of the enzyme within the cell, and finally the relation of tea oxidase to the respiration of the tea leaf.

EXPERIMENTAL

Materials and methods

Plants of the Chinese tea, *Thea Sinensis* var. *Bohea*, were used as a source of leaves for these investigations. The plants were obtained from a nursery where they had been grown from seed collected from an unknown source, probably Japan. They were grown in 5 gal. cans maintained under greenhouse conditions and grew vigorously throughout.

The bud and first two leaves of the new growth were used as a source of enzyme for all experiments unless otherwise stated. This material corresponds to that used in the preparation of ordinary teas. The leaves were ground in phosphate buffer and enzyme preparations made in a variety of ways detailed below. All operations were carried out at 0° in order to minimize inactivation of the enzyme.

Oxidase activity was determined by the measurement of oxygen uptake in the Warburg respirometer. The enzyme preparation was suspended in 2 ml. of 0.1M-phosphate at pH 5.5 in conical vessels having a total vol. of approximately 16 ml. The central well of each vessel contained 0.3 ml. of 5% KOH, while 0.3 ml. of substrate was added from the side-arm of the vessel after attainment of temperature equilibrium. A reaction temperature of 30° was used throughout.

Preparation of the active enzyme

That tea leaves contain an active oxidase may be shown as follows:

Fresh tea leaves were ground by hand in a mortar under 0.1M-phosphate buffer (pH 6.7), filtered through cheese-cloth to remove cellular debris and the filtrate centrifuged at high speed for 30 min. The clear yellowish supernatant liquid contains chiefly the tea tannins, sugars, and soluble nitrogenous compounds including the cytoplasmic proteins, and exerts little enzymic activity toward catechol (see below). The precipitate from the centrifugation was washed with buffer, recentrifuged, and this repeated four times to remove completely the tea tannins. Microscopic observation showed that this preparation consisted largely of chloroplasts and of chloroplastic material, especially

the constituent grana or green particles of which the plastid is formed. Small amounts of cell wall material, crystals of calcium oxalate, and leaf hairs were also present.

Fig. 1. Rate of oxidation of catechol and of *p*-phenylenediamine by preparations from tea leaves. A. Oxidation of catechol by the insoluble (upper curve) and the soluble (lower curve) portions of ground tea leaves. B. Oxidation of *p*-phenylenediamine by the soluble (upper curve) and insoluble (lower curve) portion of ground tea leaves.

From the standpoint of the leaf proteins, the purified precipitate represents what has been termed by Chibnall (1939) the chloroplastic fraction, while the cytoplasmic proteins are present in the original supernatant liquid. Samples of each fraction were tested for activity in the oxidation of catechol and of *p*-phenylenediamine. It may be seen (Fig. 1) that the chloroplast-grana fraction rapidly oxidizes catechol but has little effect on *p*-phenylenediamine, while with the cytoplasmic fraction just the reverse is true. It might at first be thought that two kinds of enzyme systems are here involved, namely a polyphenol-like oxidase located in the chloroplasts, and *p*-phenylenediamine oxidase or oxidase system located in the cytoplasm. It can be shown, however, that the oxidation of *p*-phenylenediamine is non-enzymic and persists even with cytoplasmic preparations which have been heated to 100° (Table 1).

Table 1. *Non-enzymic nature of p-phenylenediamine oxidation by the soluble fraction of tea leaf*

Substrate	O ₂ consumption (μ l./vessel/60 min.)	
	Untreated preparation	Boiled preparation
<i>p</i> -Phenylenediamine	83.1	94.0
Catechol	2.6	0.0

It is possible that the oxidation of *p*-phenylenediamine by tea leaves as described by Roberts (1941*b*) and as observed here represents merely a non-enzymic reaction between the tea tannins or other oxidizing agents and the *p*-phenylenediamine.

Repeated attempts were made to obtain a soluble oxidase from the supernatant liquid by precipitation, on the supposition that the presence of such an enzyme might be masked due to inactivation by the tea tannins. Precipitation with ammonium sulphate or with acetone failed to yield any fraction active in oxidizing catechol or *p*-phenylenediamine. It must be concluded, therefore, that the tea oxidase is not present in the cytoplasmic fraction, but is present rather in the chloroplastic fraction. That the oxidase is present in the chloroplast itself is strongly indicated by the experiment of the following section.

Oxidase activity of intact plastids

In order to establish the association of oxidase activity with the chloroplasts, a procedure for obtaining intact chloroplasts similar to that described by Granick (1938) was used. In this procedure the leaves are ground in an isotonic sugar solution in order to avoid the bursting of the plastids which occurs in hypotonic solutions.

Fresh tea leaves were ground under a 10% (w/w) glucose solution for 90 sec. in a blender. The resultant *brei* was filtered through cheese-cloth to remove unground leaf

fragments and cell walls, after which the filtrate was centrifuged (2000 r.p.m. in an International clinical centrifuge). The precipitate which contained, in addition to intact chloroplasts, a few grana from broken plastids, calcium oxalate crystals, etc., was again taken up in 10% glucose and recentrifuged at low speed (approximately 500 r.p.m. in an International clinical centrifuge). At this low speed the large plastids could be separated from the grana and other smaller particles.

Repetition of this procedure five times resulted in a preparation which microscopic examination revealed to consist exclusively of intact chloroplasts. Such chloroplasts actively oxidize catechol, as is shown in Table 2. Furthermore, the enzymic

Table 2. *Oxidase activity of intact chloroplasts of tea leaves together with the activity of grana derived from such chloroplasts*

	O ₂ uptake (μ l./ $\frac{1}{2}$ hr./mg. enzyme) at			
	$\frac{1}{2}$ hr.	1 hr.	1 $\frac{1}{2}$ hr.	2 hr.
Original leaf extract in 10% (w/w) glucose solution	1.21	1.09	0.80	0.80
Purified intact plastids	2.17	3.90	3.90	4.33
Plastids ground with quartz sand (granas)	4.09	3.87	3.44	3.01
Supernatant granas from purified intact plastids	1.98	2.10	1.73	1.48
Hand-ground leaf extract	6.46	5.70	4.37	3.80
Acetone-purified hand-ground leaf extract	7.29	7.02	5.67	4.86

activity of the pure intact chloroplast preparation is greater on a dry weight basis than the activity of the original whole precipitate, including chloroplasts, grana and other cellular material. It would appear probable, therefore, that the catechol oxidase activity is actually associated with the chloroplast rather than with some other kind of cellular particle.

Many methods of obtaining grana from intact chloroplasts were attempted. The most successful consisted in grinding the chloroplast preparation with quartz sand under buffer. The quartz sand was then centrifuged from the grana suspension by low-speed centrifugation. Table 2 shows that when the chloroplasts are so treated the resultant grana have an oxidase activity toward catechol even greater than that of the original chloroplast. In addition, the gradual increase in reaction rate with time, observed with intact chloroplasts, is not observed with the grana preparation. Whether the permeability of the intact plastid to catechol gradually increases with time or whether the lag in attainment of maximum rate merely represents the time needed for inward diffusion of the substrate is not revealed by these experiments. Table 2 includes for comparison a

preparation made by grinding of leaves by hand in a mortar. In this case, which is similar to that recorded in the experiment of Fig. 1, the insoluble enzyme fraction consists mainly of chloroplastic grana. The oxidase activity of the preparation is comparable to that of the grana obtained from intact plastids.

It can be concluded then that an active insoluble oxidase is contained in the chloroplasts of tea leaves and that this enzyme is associated with the grana.

The following experiment indicates, however, that the enzyme is not directly connected with the chlorophyll of the grana. A purified grana preparation was extracted with aqueous 40% (v/v) acetone. This treatment removed the bulk of the chlorophyll from the grana. The residual particles possessed as great or greater oxidase activity than the original intact grana (Table 3). This observation is in agreement with that made by Lamb & Sreerangachar (1940) who prepared the tea oxidase by a procedure involving a preliminary grinding of the leaf in aqueous acetone.

Table 3. *Effect of acetone extraction of tea oxidase on activity toward catechol*

(Each vessel contained 3 mg. catechol.)

	O ₂ consumed (μ l./hr./vessel)	
	Exp. 1	Exp. 2
Enzyme preparation	29.5	32.0
Same preparation after extraction with 40% (v/v) acetone	37.0	41.2

It has not been possible to break down the grana further and to release quantitatively the oxidase in soluble form. It has, however, been possible to obtain small amounts of soluble oxidase by repeated extraction of dried grana as is shown in Table 4.

Table 4. *Conversion of the insoluble tea oxidase into soluble form by drying in vacuo*

(All determinations made in 0.1M-phosphate, pH 5.5, with 3 mg. catechol/vessel.)

Preparation	O ₂ consumed (μ l./hr./10 mg. original enzyme)
10 mg. of whole dried acetone- extracted grana	32
1st extract of 10 mg. grana (extracted 3 hr. in buffer)	30
2nd extract (3 hr.)	18
3rd extract (19 hr.)	18
Residue after 3 extractions	33

Grana preparations were taken to dryness in a vacuum desiccator at room temperature and were then extracted with repeated portions of 0.1M-phosphate, pH 5.5. As can be seen in Table 4, only a

small amount of the total oxidase was extracted in soluble form since the residual particles after extractions were still highly active.

Substrate specificity of the tea oxidase

The insoluble enzyme contained in the grana of tea leaves attacks a wide variety of phenols, including the tea tannin (hot water extract from fresh tea leaves), catechol, gallic acid and dihydroxyphenylalanine. It fails to attack monophenols such as *o*- and *p*-cresol and tyrosine. It does not attack *p*-phenylenediamine at any considerable rate.

Table 5. *Specificity of tea oxidase*

(Acetone extracted, vacuum dried crude grana as enzyme. Concentration of all substrates: 1.5 mg./ml.)

Substrate	O ₂ consumption (μ l./10 mg. enzyme/hr.)
None	4.4
Catechol	72.5
Phloroglucinol	7.7
Dihydroxyphenylalanine	44.3
<i>o</i> -Cresol	5.3
<i>p</i> -Phenylenediamine	10.8
<i>p</i> -Cresol	0.0
Gallic acid	17.4
<i>p</i> -Hydroxybenzoic acid	4.3
Tyrosine	0.0

Inhibition of tea oxidase

The action of the tea oxidase on catechol is relatively insensitive to cyanide, 0.001M-KCN exerting but little effect on the rate of the oxidation. Tea oxidase is inhibited by low concentrations of *p*-nitrophenol, a substance which has been shown to inhibit the polyphenol oxidase of spinach leaves (Bonner & Wildman, 1946); 1 mg./ml. of this substance caused 86% inhibition in the rate of oxidation of catechol, while one-tenth of this concentration still brought about a significant depression in rate (Table 6).

Table 6. *Effect of various inhibitors on tea oxidase*

(Substrate, 3 mg. catechol/vessel in all cases. 10 mg. acetone-extracted whole grana/vessel.)

Inhibitor	O ₂ consumption (μ l./10 mg. enzyme/hr.)	Inhibition (%)
None	39.9	—
KCN, 0.01M	7.1	82.3
KCN, 0.001M	38.1	4.5
None	72.8	—
<i>p</i> -Nitrophenol: 1 mg./ml.	19.5	73.0
<i>p</i> -Nitrophenol: 0.1 mg./ml.	42.6	41.0

Relation of tea oxidase to normal leaf respiration

The respiration of excised tea leaves was studied with preparations which had been chopped into fragments approximately 4 sq.mm. in area. The leaf

fragments (0.1 g.) were then weighed into each Warburg vessel, 2 ml. of 0.1 M-KH₂PO₄, pH 4.5 added, and gas exchange studied in the usual manner. The penetration of added substrates or inhibitors into the leaf was assured by twice repeated vacuum infiltration of the leaf fragments in the test solution. Added catechol was without significant effect on O₂ uptake or the CO₂ evolution of tea leaves (Table 7). This behaviour is in marked

Table 7. *Effect of catechol on the respiration of tea leaves*

(Leaves vacuum-infiltrated with catechol; 100 mg. leaf fragments (c. 4 sq.mm.) in 2.0 ml. 0.1 M-KH₂PO₄, pH 4.5.)

Substrate	Gas	Gas exchange (μ l./100 mg. leaf/hr.)	
		Exp. 331	Exp. 330
None	O ₂	77	58
	CO ₂	69	53
Catechol (1 mg./ml.)	O ₂	80	57
	CO ₂	70	60

contrast to that of spinach leaves in which polyphenol oxidase is the terminal respiratory oxidase (Bonner & Wildman, 1946). These rapidly oxidize catechol, while at the same time CO₂ evolution by the leaf is greatly depressed. It would appear that in tea leaves, on the contrary, added catechol does not penetrate to the site of the tea oxidase, and it is unlikely that this oxidase participates in respiration, particularly since, as has also been shown in the work with spinach leaves, other respiratory enzymes such as the dehydrogenases probably occur among the cytoplasmic proteins. Potassium cyanide in the concentration of 0.001 M, which has been shown above to be without effect on the tea oxidase in its action on catechol, has some inhibiting effect on normal tea-leaf respiration; *p*-nitrophenol, on the other hand, is less inhibitory to respiration than to the tea oxidase (Table 8). These facts taken in conjunction make it appear improbable that the tea oxidase participates in the normal respiration of tea leaves.

Table 8. *Effect of inhibitors on the rate of respiration of tea leaves*

(Inhibitors infiltrated into leaves suspended in 0.1 M-KH₂PO₄, pH 4.5.)

Addition	O ₂ uptake (μ l./100 mg. leaf/hr.)		Inhibition in 2nd hr. (%)
	1st hr.	2nd hr.	
None	72	66	—
KCN, 0.001 M	49	43	35
<i>p</i> -Nitrophenol: 1 mg./ml.	50	38	42
<i>p</i> -Nitrophenol: 0.1 mg./ml.	67	64	3

DISCUSSION

The enzyme responsible for the oxidation of the tea tannins and the fermentation of black tea resembles polyphenol oxidase in that it can carry on direct oxidation of catechol and related *o*-diphenols. The polyphenol oxidases which have been studied extensively, such as those of the potato tuber (Kubowitz, 1937), mushrooms (Graubard & Nelson, 1935), and spinach leaves (Bonner & Wildman, 1946), are all readily soluble proteins occurring in the cytoplasm of the plant, and in spinach leaves polyphenol oxidase is definitely absent from the chloroplasts. In tea leaves, on the contrary, the oxidase appears to be normally absent from the cytoplasm and to occur solely in the chloroplasts. The enzyme does not depend on the chlorophyll, since removal of this material with acetone leaves the oxidase activity of the plastid or grana undiminished. The enzyme is however closely associated with the structure of the grana, and it has not been possible quantitatively to extract or remove the oxidase from the grana. This fact accounts for the insolubility of the enzyme, reported by earlier workers. Tea oxidase is but one of a series of enzymes (e.g. succinic dehydrogenase, cytochrome oxidase, etc.), whose activity is associated with the structure of the cell. In the present case, however, insolubility of the enzyme can be attributed to the anatomic aspects of its distribution within the cell. The enzymic activities of isolated chloroplasts and of grana have been but meagrely investigated. Neish (1939) has reported that isolated grana of *Trifolium* and other chloroplasts exhibit catalase and carbonic anhydrase activity.

Polyphenol oxidase has been shown to be the terminal oxidase in the respiration of the potato tuber (Baker & Nelson, 1943; Boswell & Whiting, 1938) and in the spinach leaf (Bonner & Wildman, 1946). In tea, however, it would appear that the normal leaf respiration does not involve polyphenol oxidase since respiration remains unaffected either by inhibitors of the enzyme or by the addition to the leaf of substrates such as catechol. The normal leaf respiration of tea must therefore involve other and as yet unrecognized oxidase systems. Clearly also the tea tannins which are present in the intact cell do not normally come in contact with the tea oxidase. Contact between tannin and enzyme in tea fermentation is presumably brought about by the preliminary crushing to which the leaves are subjected.

SUMMARY

1. The enzyme of tea leaves which is responsible for the oxidation of the tea tannins in tea fermentation is an oxidase having substrate specificity resembling that of polyphenol oxidase.

2. The tea oxidase, unlike the polyphenol oxidase of other tissues, is normally insoluble in water or buffer solution. The oxidase activity is associated with the chloroplasts of the leaf. If the chloroplasts are broken so as to release the grana it may be shown that the oxidase is associated with the grana.

3. Removal of chlorophyll from the grana by extraction with acetone leaves the enzyme activity of the preparation unaffected. Soluble oxidase may be extracted in small quantities from dried grana preparations.

4. The tea oxidase does not appear to participate in the normal leaf respiration of the tea plant.

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The Assay of the Vitamin B₆ Complex

By R. H. HOPKINS AND R. J. PENNINGTON

Department of Brewing and Industrial Fermentation, University of Birmingham

(Received 7 June 1946)

It is now known that pyridoxal, pyridoxamine and pyridoxal 'phosphate' (the co-enzyme of certain amino-acid decarboxylases) occur naturally and contribute to the 'vitamin B₆' effect by which pyridoxin was originally recognized. These compounds, closely chemically related to pyridoxin, give quantitatively very different responses with certain micro-organisms when these are used as the biological test object, while they have approximately equal potencies for the rat and the chick (Snell & Rannefeld, 1945; Luckey, Briggs, Elvehjem & Hart, 1945), and, presumably, for human beings. It is therefore convenient, if not essential, to have a microbiological assay method in which the test organism responds equally to all the compounds of the vitamin B₆ complex, so that assays of food-stuffs may be made which will give values proportional to their total content of vitamin B₆-active substances.

The yeast *Saccharomyces carlsbergensis* 4228, used by Atkin, Schultz & Frey (1943) for the microbiological determination of pyridoxin appears to fulfil this condition. Snell & Rannefeld (1945), using this yeast, found that the relative activities of pyri-

doxin, pyridoxal and pyridoxamine were 1.00 : 1.16 : 1.09, while Miller & Baumann (1945) found the activities equal within $\pm 5\%$. The method of assay with *S. carlsbergensis* has been used by us with slight modifications and tested for a variety of foodstuffs. The existence of an unknown member or members of the vitamin B₆ complex postulated by Melnick, Hochberg, Himes & Oser (1945) has not been confirmed by data presented here. The better-known chemical methods for the determination of vitamin B₆ have been tested and some criticisms are offered.

EXPERIMENTAL

Methods

The procedure of Atkin *et al.* (1943) was followed closely, only departures from it are stated here.

Culture. We are indebted to Dr Frey for a culture of *S. carlsbergensis* 4228. Another strain of *S. carlsbergensis* (Frohberg strain) kept in these laboratories was only stimulated slightly by pyridoxin.

Standards. Pyridoxin hydrochloride was kindly supplied by the Medical Research Council (a commercial sample was